1018-Pos Board B773
VMD as a Software for Visualization and Quantitative Analysis of Super Resolution Imaging and Single Particle Tracking
Yanxin Liu[1,2], John E. Stone[2], En Cai[1], Jingyi Fei[1], Sang Hak Lee[1], Seongjin Park[1], Taekjip Ha[1], Paul R. Selvin[1], Klaus Schulten[1,2].
[1]Department of Physics and Center for the Physics of Living Cells, University of Illinois at Urbana-Champaign, Urbana, IL, USA, [2]Beckman Institute, University of Illinois at Urbana-Champaign, Urbana, IL, USA.
Super resolution imaging and high-precision single particle tracking are promising techniques to study biomolecular trafficking and localization as well as intracellular structures. The tremendous amount of data acquired from the experiments pose a great visualization and analysis challenge. VMD, a software for visualizing and analyzing trajectories from molecular dynamics simulations, has the ability to handle hundreds of millions of particles and hundreds of thousands of frames in a time-dependent manner. We extended VMD with new functions for handling data from super-resolution imaging and high-precision single particle tracking experiments and performed visualization and data analysis. The analysis can take advantage of VMD's existing capability to display particles in various representations and to compute system properties employing GPUs. The Tcl/Tk interface in VMD adds a layer of flexibility to implement project-specific functionality specified by users. The use of VMD is demonstrated for two examples: (1) employing STORM to reveal the organization of long non-coding RNAs in nuclear bodies; (2) combining single particle tracking and PALM to study AMPA receptor trafficking at live synapses.

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Super Resolution Mapping of Adhesion Molecules in Confined Cellular Environments using Monomeric Streptavidin Ligands
Ingrid Chamma[1], Olivier Rossier[1], Kok Hong Lim[2], Isabel Gauthereau[1], Gregory Giannone[1], Sheldon Park[3], Daniel Choquet[1], Matthieu Sainlos[1], **Olivier R. Thoumine**[1].
[1]Interdisciplinary Institute for Neuroscience, Bordeaux, France, [2]Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD, USA, [3]University at Buffalo, Buffalo, NY, USA.
High resolution tracking of individual cell surface proteins is limited by several factors, including probe size that can impair protein trafficking and restrict their access to crowded cellular environments, ligand multivalency that induces protein cross-linking, and potential loss of function of recombinant proteins fused to large tags. To overcome these issues, we developed a labeling method that uses monomeric streptavidin (mStrav) conjugated to highly photo-robust organic Atto dyes. mStrav is a 3-nm-molecule, stable and easy to produce, that binds biotinylated proteins with high affinity (Lim et al., Biochemistry 2011). Atto-conjugated mStrav is then used as a probe to label cells co-transfected with a membrane molecule of interest carrying a 15 amino acid extra-cellular acceptor peptide (AP), and the biotin ligase BirA that covalently adds biotin to the tag during protein maturation. We used this technique to study membrane diffusion and nanoscale organization of the synaptic adhesion molecules neurexin-1β and neuroligin-1 in hippocampal neurons and β3-integrins in fibroblasts. We show that the monovalency of mStrav prevents protein cross-linking, allowing more accurate diffusion measurements. The small sizes of the AP tag (<1nm) and mStrav (~3nm) do not perturb protein function and allow access to single molecule dynamics and high resolution mapping in confined regions such as synapses or cell-matrix contacts. We also used mStrav along with nanobodies (~3nm) for dual-color single molecule tracking. Moreover, the binding of mStrav can be reversed with excess biotin, a property that can be used to study protein endocytosis and recycling. Finally, the photostability of Atto dyes conjugated to mStrav allows for long trajectory recordings and live STED imaging. Taken together, monomeric streptavidin is a simple and versatile tool that can be used to track an arbitrary membrane protein in living cells.

1020-Pos Board B775
Diffusion Mapping in Living Cells using Camera-Based Correlation Spectroscopy and Phasor Analysis
Per Niklas Hedde, Enrico Gratton.
Laboratory for Fluorescence Dynamics, Biomedical Engineering Department, University of California Irvine, Irvine, CA, USA.
Image correlation spectroscopy (ICS) is a powerful tool to study dynamics in living cells and tissues. However, when using a single point detector, the mapping of molecular movement is either slow or limited to a small field of view due to the sequential, point-by-point data acquisition. With an area detector such as a camera on the other hand, molecular motion can be captured simultaneously for each image pixel, allowing a fast mapping of the entire structure under study.
Usually, image data acquired with a camera is analyzed by correlating the fluorescence time trace captured in each pixel followed by fitting a function modeling the dynamic process. Yet, with ~2^{16} data points at hand, manual data evaluation is impossible while automatic fitting procedures are prone to errors because they rely on careful adjustment of start parameters and constrains. In particular, a fit to correlation spectroscopy data from regions containing static molecules or no molecules at all produces inconclusive results.
In fluorescence lifetime imaging (FLIM), the phasor approach is a popular method to obtain a graphical representation of the spatial distribution of lifetimes [1]. Phasor-based analysis is fast and does not require any fitting of the data. We applied the phasor method to camera-based correlation spectroscopy resulting in a map of molecular diffusion. This map can be overlaid with the intensity image to quickly identify spatial variations of the diffusion of fluorescently labeled biomolecules within living cells.
Work supported in part by NIH grants P50 GM076516 and P41 GM103540.
[1] M. Digman et al. *The phasor approach to fluorescence lifetime imaging analysis.* Biophysical Journal 94 (2008), L14-L16.

1021-Pos Board B776
Mapping Diffusion in a Living Cell using the Phasor Approach
Suman Ranjit, Luca Lanzano, Enrico Gratton.
Laboratory for Fluorescence Dynamics, Biomedical Engineering, University of California Irvine, Irvine, CA, USA.
The diffusion of a fluorescent protein within the cell has been measured by either using the fluctuation based techniques (FCS, RICS) or through particle tracking. However none of these methods enable us to measure the diffusion of the fluorescent particle at each pixel of the image of the cells. Measurement using the conventional single point FCS at each pixel of the image results in the continuous exposure of the cell to the laser and eventual bleaching of the sample.
To overcome this limitation we developed a new method of scanning while constructing fluorescent image of the cell. In this new method of scanning, the intensity trace at each pixel is collected multiple times before it moves to the next pixel. Alternatively, while acquiring the image, the laser scans each individual line multiple times before moving to the next line. This continues until the complete area is scanned. This is different from the RICS approach where the data is acquired by scanning each line once and scanning the image multiple times. The total time of data acquisition needed for this method is shorter than the time required for the traditional FCS analysis. However, a single pixels the time sequence is relatively small, requiring a non-conventional analysis of the correlation function to extract information about the diffusion and the number of molecules.
These intensity data has been analyzed using the phasor approach that was originally created for the analysis of FLIM data. Analysis using this method results in calculation of diffusion constant of the fluorescent species at each pixel of the acquired image, and thus the diffusion map inside the cell can be created. This enables this new technique to distinguish mobility at every pixel of the image.
Funding - P50GM076516 P41GM103540.

1022-Pos Board B777
Focal Adhesion Axial Topography by the Z-Phasor Approach in Confocal Microscopy
Enrico Gratton, Chili Chiu.
University of California, Irvine, Irvine, CA, USA.
The protein lateral and axial organization within focal adhesions has been studied by state of the art super resolution methods due to its thin structure, well below diffraction limit. However, to achieve high axial resolution, most of the current approaches rely on either sophisticated optics or diligent sample preparation requiring sample fixation that limits their application. In this report we present a phasor-based method that can be applied to fluorescent samples to determine the precise axial position of proteins using a conventional confocal microscope. We demonstrate that when about a total of 4000 photon counts are collected along a z-scan, axial localization precision close to 10 nm is achievable. We show at 10nm resolution that axial localization of paxillin, FAK and talin is similar at different focal adhesion sites, while F-actin shows a sharp increase in height towards the cell center. We further demonstrated that using line scans we can obtain axial resolution of 10 nm with a temporal resolution of less than a second of the changes of topography along the line. With the advantage of simple data acquisition and no special instrument requirement, this approach could have wide dissemination and application potentials.
Work supported in part by NIH grants P50 GM076516 and P41 GM103540.